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The R336Q mutation in human mitochondrial EFTu prevents the formation of an active mt-EFTu·GTP·aa-tRNA ternary complex

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ABSTRACT

The mitochondrial translational machinery allows the genes encoded by mitochondrial DNA (mtDNA) to be translated in situ. Mitochondrial translation requires a number of nucleus-encoded protein factors, some of which have been found to carry mutations in patients affected by mitochondrial encephalomyopathies. We have previously described the first, and so far only, mutation in the mitochondrial elongation factor Tu, mt-EFTu, in a baby girl with polycystic encephalopathy, micropolygyria, and leukodystrophic changes. Despite that the mutant mt-EFTu was present in normal amount in the patient's tissues, mitochondrial translation was severely reduced, determining multiple defects in the amount and activity of mtDNA-dependent respiratory chain complexes. By an *in-vitro* reconstructed translational system, we here provide evidence that the mutant mt-EFTu variant fails to bind to aminoacylated mitochondrial tRNAs, thus explaining the observed impairment of mitochondrial translation. This is the first analysis on the molecular mechanism of a mtDNA translation defect due to a nuclear gene mutation.

1. Introduction

Mammalian cells have two distinct translation systems, cytosolic and mitochondrial, the latter being closer to that of prokaryotes, in agreement with the ancestral origin of mitochondria. The mitochondrial system allows the in situ translation of genes encoded by mitochondrial DNA (mtDNA). In addition to tRNAs and rRNAs encoded by, and transcribed from, mtDNA itself, the mitochondrial translational machinery requires a number of nucleus-encoded protein factors, which are translated in the cytosol and imported into mitochondria [1].

The translation process is organized in 3 phases: (i) initiation, where messenger RNA binds to the ribosome; (ii) elongation, where the nascent peptide is actually synthesized; and (iii) termination, where the process ends and the ribosome can be released from the mRNA.

The elongation phase requires the concerted action of (at least) three mitochondrion-specific factors: (i) mitochondrial elongation factor 1 (mt-EFG1), which allows the nascent peptidyl-tRNA to move from the A to P sites of the ribosome; (ii) mitochondrial elongation factor Tu (mt-EFTu), which forms a ternary complex with GTP and aminoacylated (aa)-tRNA and promotes the codon-directed binding of the aa-tRNA to the A-site of the ribosome.; and (iii) mitochondrial elongation factor Ts (mt-EFTs), which promote the exchange of guanine nucleotide on mt-EFTu, thus promoting the catalytic use of this factor.

Human mt-EFTu [2] is encoded by a 9-exon gene located on chromosome 16p11.2 (an intronless pseudogene is on 17q11.2 [3]). The crystallographic structure of bovine mt-EFTu-GDP [4] shows the presence of three domains, I— a Mg^{++}-GTPase domain, II— an aa-tRNA binding domain, and III— a mt-EFTs binding domain. The structure of mt-EFTu is similar to that of *Escherichia coli* and *Thermus aquaticus*, but the mitochondrial protein contains an 11-aa C-terminal extension that is hypothesized to increase its affinity to the mt-tRNA species, which are shorter than cytosolic tRNAs [4,5]. In a dynamic reconstruction of the mt-EFTu mechanism, domains II and III are relatively fixed, whereas domain I rotates in the mt-EFTu-GDP vs. mt-EFTu-GTP states [6,7]. Mt-EFTu is recycled by mt-EFTs, which replaces GDP with GTP. Mt-EFTu-GDP binds to mt-EFTs, thus altering the Mg^{2+} binding site and reducing the affinity of mt-EFTu for guanine nucleotides [8]. Mt-EFTs is then released and a new GTP molecule is bound to mt-EFTu.

In the last years the number of mitochondrial translation defects found in human patients that are due to mutations in nucleus-encoded factors has expanded, including mutations in mt-EFG1 [9,10,11], MPRS16 [12], mt-EFTs [13], MPRS22 [14] and different mitochondrial aminoacyl-tRNA synthetase (for a complete review see ref. [15]).

We previously described the first, and so far only, mutation in mt-EFTu [11], in a baby patient with polycystic encephalopathy, micropolygyria, and leukodystrophic changes. We provide here mechanistic evidence, which explains impaired mitochondrial translation by the mt-EFTu mutant variant.

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2. Materials and methods

2.1. Expression and purification of human mt-EFTu in E. coli

2.1.1. Cloning in a procaryotic expression vector

According to the protein sequence deduced by the NCBI annotation NM_003321, the mutation in our patient determines a change of the arginine at position 339 into a glutamine. However, the human mt-EFTu protein sequence deduced from NM_003321 contains two methionine residues in the N-terminus, one at position +1, the other at position +4. In all other mammalian species the first methionine residue (M1) is missing, and the protein starts at the position corresponding to human M4. It is very likely that in fact the true human mt-EFTu protein is three-residue shorter than the sequence predicted by NM_003321. This redefinition of the primary structure of human mt-EFTu is indeed found in the Expasy-Swissprot database (accession no. P49411). Accordingly, we will redefine the protein sequence of human mt-EFTu as corresponding to the P49411 annotation, and as a consequence the R339Q will be replaced by R336Q. The cDNA encoding the putative human mt-EFTu^{wt} mature form, tagged with a hexahistidine (His) stretch at the C terminus, was cloned into the procaryote expression vector pET24(a) (Novagen). The cDNA corresponding to NCBI NM_003321 was first PCR-amplified by high-fidelity Taq Gold (Invitrogen) with modified primers containing

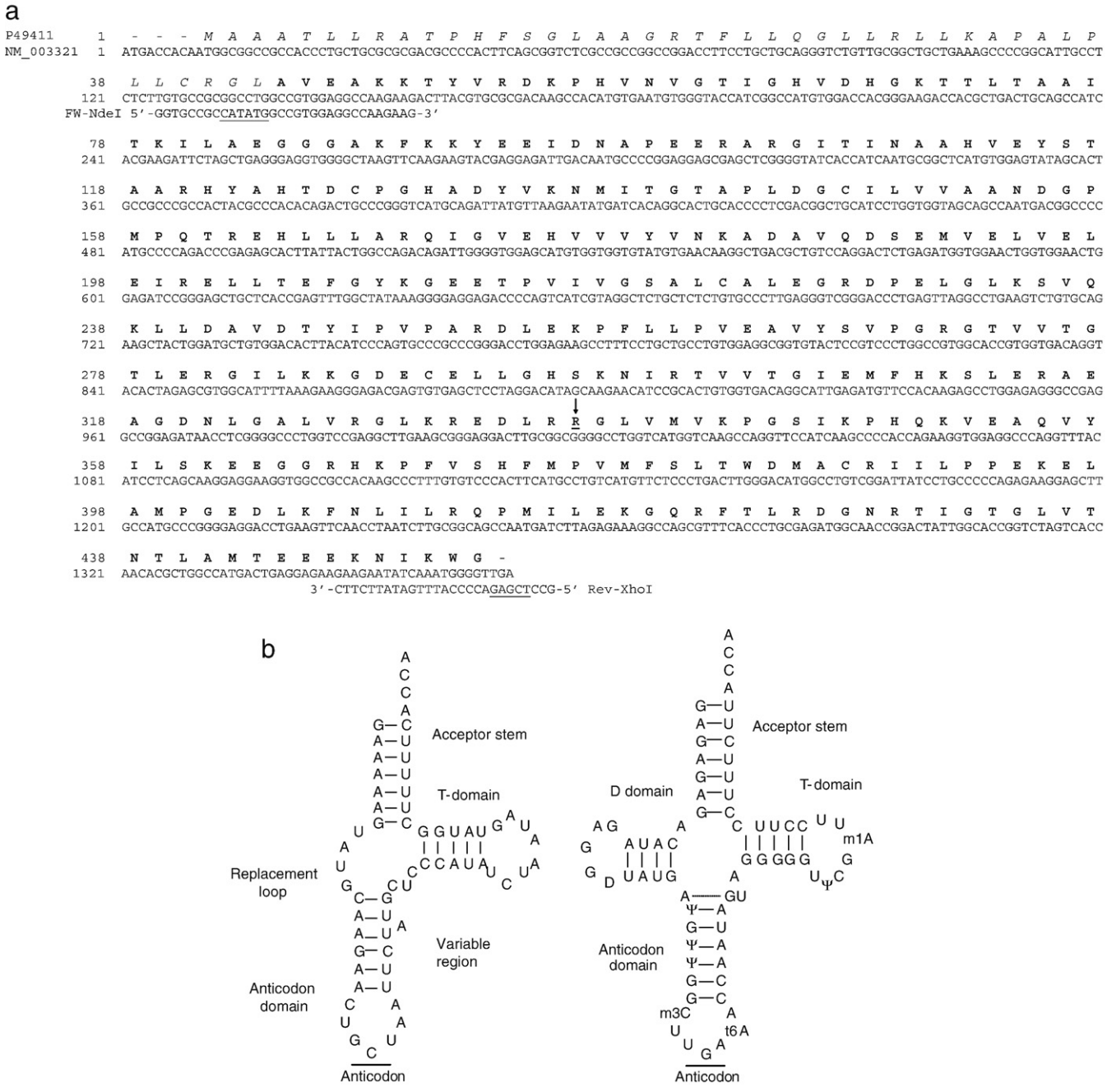

Fig. 1. Structures and sequences of human mt-EFTu and bovine mt-tRNA^{Ser} species used in this study. (Panel a) Human mt-EFTu aminoacid sequence. The putative mitochondrial targeting sequence is in italic. An arrow indicates the R336 residue. Modified forward (FW-NdeI) and reverse (Rev-XhoI) primers used for cDNA cloning are aligned along the nucleotide sequence. The sequence corresponding to the restriction sites are underlined. (Panel b) Bovine mitochondrial tRNA^{Ser} primary and secondary structures.

a NdeI restriction site (forward primer, Fw) and a XhoI restriction site (reverse primer, Rev), as follows:

Fw (NdeI) 5′-GGTGCCGCCATATGGCCGTGGAGGCCAAGAAG-3′
Rev (XhoI) 5′-GCACTCGAGACCCCATTTGATATTCTTC-3′.

PCR conditions were: hot start 95 °C 10′, 25 cycles of denaturation 95 °C 45″, annealing 56 °C 1′, elongation 72 °C 2′30″, and a last extension cycle 72 °C 5′. The cDNA fragment encodes a protein missing the first 43 N-terminal aminoacids of the protein sequence P49411. We also modified the forward primer in order to introduce an initiation methionine at position +1 of the recombinant protein (Fig. 1a). Both the PCR insert and the pET24(a) were digested with NdeI and XhoI restriction enzymes, ligated using T4 DNA ligase (Invitrogen) for 1 h at 26 °C, and used to transform competent cells. The insert was verified by sequence analysis. The cDNA encoding mature EFTu^{R336Q} was obtained by directed mutagenesis (Stratagene) using the protocol recommended by the manufacturer and the following modified primers:

Fw 5′-GGGAGGACTTGCGGCAGGGCCTGGTCATGGT-3′
Rev 5′-ACCATGACCAGGCCCTGCCGCAAGTCCTCCC-3′.

2.1.2. Expression of recombinant mt-EFTu in E. coli

After transformation with the suitable recombinant plasmids, Rosetta(DE3)pLysS *E. coli* competent cells (Novagen) were grown to mid-log phase in 2 l of LB media with 25 μg/ml kanamycin and 34 μg/ml chloramphenicol at 37 °C under constant agitation, and recombinant protein expression was induced with 0.4 mM IPTG at 25 °C for 4 h.

2.1.3. mt-EFTu purification

Cells expressing recombinant, His-tagged, mature mt-EFTu^{wt} and mt-EFTu^{R336Q} were collected by centrifugation at 5860 ×*g* for 15′ at 4 °C, resuspended in lysis buffer (50 mM Tris–HCl pH 7.5, 100 mM KCl, 10 mM $MgCl_2$), centrifuged again at 5860 ×*g* for 15′ at 4 °C, and pellets were fast-frozen in liquid nitrogen. The frozen cells were kept on ice, resuspended with 50 mM Tris–HCl pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, 10% glycerol, 7 mM 2-β-mercaptoethanol, 0.2 mM phenylmethanesulfonyl fluoride PMSF, 0.1% Triton X-100, and sonicated with a Brandson 450D sonicator on ice. Samples were ultracentrifuged at 100,000 ×*g* for 1 h at 4 °C in a Beckman ultracentrifuge in a fixed rotor. The proteins were then purified by chromatography through a His-TrapTM HP column (GE Healthcare) by using a FPLC Amersham bioscience AKTA primer purifier, injected with 50 mM Tris–HCl pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, 10% glycerol, 7 mM 2-β-mercaptoethanol, 10 μM GDP and eluted with 500 mM imidazole (5% to 100%). The fractions enriched with recombinant EFTu^{His} proteins were collected, dialyzed with a Slide-A-Lyzer dialysis cassette (cutoff at 10,000 Da), in 20 mM Tris–HCl pH 7.6, 40 mM KCl, 1 mM $MgCl_2$, 10% glycerol, 6 mM 2-β-mercaptoethanol at 4 °C. The purified proteins were stored at −80 °C until use.

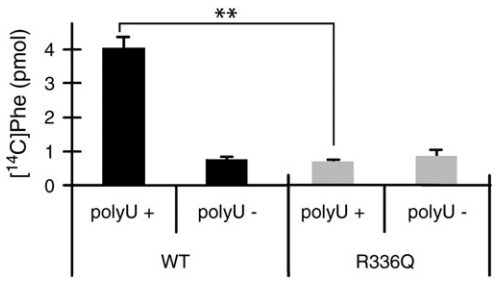

Fig. 2. *In-vitro* translation assay. The histograms display the amount of TCA-precipitable [^{14}C]Phe expressed in pmol, produced in 30′ in the presence (+) or absence (−) of translable poly(U) RNA, and of mt-EFTu^{wt} (WT) or mt-EFTu^{R336Q} (R336Q) recombinant proteins. The bars represent ± standard deviation. Black bars refer to experiments with WT, while the grey bars refer to experiments with R336Q mt-EFTu species. Student's *t*-test analysis: $^{**}p<0.01$.

Table 1
In-vitro translation assay.

EFTu	polyU	[^{14}C]Phe(pmol) ± SD
1 μM EFTu WT	+	4.027 ± 0.350
	−	0.740 ± 0.067
1 μM EFTu R336Q	+	0.725 ± 0.014
	−	0.852 ± 0.014
MIX: 0.5 μM EFTu WT + 0.5 μM EFTu R336Q	+	3.229
	−	0.787

Data represent the amount of TCA-precipitable [^{14}C]Phe expressed in pmol ± standard deviation, produced in 30′ in the presence (+) or absence (−) of poly(U) RNA, and of mt-EFTu^{wt} (WT) or mt-EFTu^{R336Q} (R336Q) recombinant proteins or both WT and R336Q in ratio 1:1.

2.1.4. Circular dicroism

The far-UV (200–260 nm) CD spectra of the protein were measured by a J-720 automatic spectropolarimeter (Japan Spectroscopic Co.) at 10 °C. The buffer was 0.4 mM Tris–HCl 7.4, 40 mM KCl, 1 mM $MgCl_2$, 0.2% Glycerol and 0.001% 2-β-mercaptoethanol. The protein concentrations were 0.049 mg/ml of wild-type and 0.027 mg/ml of mutant. The percentages of mt-EFTu fraction were estimated by SDS-PAGE.

2.2. mt-tRNA aminoacylation

Native tRNA for mitochondrial Serine, UGA and GCU, were purified from bovine liver as previously described [16]. Recombinant Ser-mt-tRNA Synthetase was a kind gift of Prof. Kimitsuna Watanabe. Aminoacylation was performed using the method described by Pingoud et al. [17]. Briefly tRNAs were incubated in 100 μl with 100 mM Hepes pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, 1 mM Spermine, 2 mM ATP, 1 mM DTT, 10 μCi [^{3}H]Serine, 18 μg Ser-mt-tRNA-Synthetase and ≈0.5 μg mt-tRNA$^{Ser(GCU)}$ or mt-tRNA$^{Ser(UGA)}$, at 37 °C for 30′. Aminoacylated Ser-tRNA^{Ser} species were purified with a NAPTM column (GE Healthcare), precipitated with ethanol, resuspended in 10 mM KOAc pH 5.2 and stored at −80 °C until use. In spite that both the nucleotide sequence and the predicted secondary structure of human mt-tRNA$^{Ser(GCU)}$ and mt-tRNA$^{Ser(UGA)}$ differ greatly from each other (Fig. 1b), they both fold into a similar tertiary structure [18] and share the same mt-seryl-tRNA synthetase [19].

2.3. In-vitro translation assay

Poly(U)-directed poly-phenylalanine synthesis with Phe-mt-tRNA (Phe) was carried out in an *in-vitro* translation system consisting of purified *E. coli* ribosomes (70 S), prepared as described [20], EFG from *E. coli* [21], poly(U) and energy regeneration system, essentially as described [22,23], with slight modifications. Briefly, we mixed in a total volume of 10 μl a final concentration of 50 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 10 mM NH_4Cl, 1 mM DTT, 0.5 mM GTP, 2 mM ATP, 2.5 U/ml Pyruvate kinase, 2.375 mM Phosphoenolpyruvate, 0.5 mg/ml poly(U) RNA, 2 μM *E. coli* EFG, 1 μM recombinant EFTu, 6 μg/ml recombinant human mt-tRNA^{Phe} synthetase, 75 pmol/ml purified bovine mt-tRNA^{Phe}, 0.01 mM [^{14}C]Phe and 50 pmol/ml purified *E. coli* ribosomes. The reaction was allowed to proceed for 30′ at 37 °C. Blank reactions were carried out in parallel for each sample by omitting poly(U) in the reaction mix. Aliquots of 8 μl per sample were spotted onto nitrocellulose filters, treated with 10% trichloroacetic acid (TCA) for 60′ at 85 °C followed by cold 10% TCA for 30′. After washing in 100% ethanol, the amount of poly-[^{14}C]Phe peptides was measured in a liquid scintillation counter. The conversion of cpm into pmol of [^{14}C]Phe was obtained by constructing a linear standard plot (not shown). Values were expressed as pmol of precipitable [^{14}C]Phe.

2.4. Non-enzymatic hydrolysis protection assay

The ability of wild-type and mutant mt-EFTu (mt-EFTu^{wt}, mt-EFTu^{R336Q}) to protect Ser-tRNASer from spontaneous deacylation

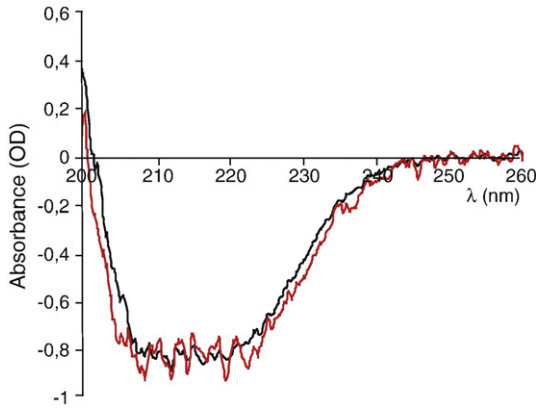

Fig. 3. Circular dicroism spectra of the purified EFTu proteins: mt-EFTu^{wt} is represented by a black line, mt-EFTu^{R336Q} by a red line.

was monitored as described [24], with slight modifications. Briefly, 10 pmol of purified EFTu^{wt} or EFTu^{R336Q} were incubated with 8 pmol of bovine aminoacylated Ser-mt-tRNA$^{(UGA)}$ or Ser-mt-tRNA$^{(GCU)}$, in 75 mM Tris–HCl pH 7.6, 75 mM NH_4Cl, 15 mM $MgCl_2$, 7.5 mM DTT, 60 μg/ml BSA, 1 mM GTP, 2.4 mM phosphoenol-pyruvate/0.1 mU pyruvate kinase (PEP/PK), in a final volume of 40 μl. Incubation was carried out at 30°C for 90 min and aliquots (8 μl) were withdrawn at various times ($t = 0'$, 30′, 60′, and 90′) and precipitated in 5% cold TCA. The precipitate was collected on nitrocellulose filters and the amount of EFTu-GTP-[^{3}H]Ser-tRNA^{Ser} was quantified using a liquid scintillation counter. The conversion of cpm into pmol of [^{3}H]Ser-tRNA^{Ser} was obtained by constructing a linear standard plot (not shown). The amount of EFTu-GTP-[^{3}H]Ser-tRNA^{Ser} was expressed as $\ln(x_t/x_0)$ versus time, where x_t is the concentration of EFTu-GTP-[^{3}H]Ser-tRNA^{Ser} at time = t and x_0 is the concentration of EFTu-GTP-[^{3}H]Ser-tRNA^{Ser} at time = 0.

2.5. Statistical analysis

Unpaired, two-tail Student's t-test was used for statistical analysis.

3. Results and discussion

In *T. aquaticus*, EFTu binds the 5′-side of the aa-tRNA acceptor stem inside a pocket formed by the three domains (I, II, III). Therefore, many residues can be involved in this important step [25]. Previous studies on both *E. coli* and bovine mt-EFTu species suggest that the wild-type R338 residue is involved in aminoacyl-tRNA binding [24], whereas the contiguous L341 residue is important for the stability of the mt-EFTu–GTP–mt-EFTs complex [26].

In order to test the functional consequences of the R336Q mutation, we expressed in, and purified from, *E. coli*, the allegedly mature forms of mt-EFTu^{wt} and mutant mt-EFTu^{R336Q}, obtained by expressing cDNAs lacking the 43 N-terminal aminoacid residues of the P49411 reference sequence for human mt-EFTu, which are predicted to encompass the mitochondrial targeting signal (Fig. 1a).

First, we tested both mt-EFTu^{WT} and mt-EFTu^{R336Q} variants in an *in-vitro* translation system, which can translate a poly(U) RNA stretch into [^{14}C] radiolabeled Phe oligopeptides. Robust *in-vitro* synthesis of poly-[^{14}C]Phe was measured with mt-EFTu^{wt}, whereas the values obtained with mt-EFTu^{R336Q} were virtually identical to those of the blank reactions, indicating severe loss of function (Fig. 2 and Table 1).

As shown in Table 1, *in-vitro* translation was also normal in the presence of a 1:1 mixture of mt-EFTu^{wt} and mt-EFTu^{R336Q}, in line with the genetic features of the disease that was inherited as a strictly recessive trait.

These results, obtained *in vitro*, are in agreement with the profound translation defect detected in vivo on fibroblasts from our mt-EFTu^{R336Q} mutant patient [11]. However, both these results could not define the mechanistic defect associated with mt-EFTu^{R336Q}, which could be due to either a conformational alteration of the protein or to functional impairment, for instance in the binding of GTP, aminoacylated-mt-tRNA, or both, to form an active mt-EFTu–GTP–aminoacyl-tRNA complex.

To gain insight into the mechanistic abnormality underlying the translational defect, we first used circular dicroism analysis on purified proteins, in order to test whether the mutant and wild-type species were structurally different. As shown in Fig. 3, the spectra obtained from the two protein species were perfectly overlapping, indicating that no conformational changes were present in mt-EFTu^{R336Q} vs. mt-EFTu^{wt}.

This result, which is concordant with previous *in-silico* structural predictions [11] and with the demonstration that mutant mt-EFTu^{R336Q} is present in normal amount in fibroblasts and tissues of our patient [11], prompted us to test whether the mutation could impair the formation of the mt-EFTu–GTP–aminoacyl-tRNA complex, as suggested by previous studies in procaryotes [24,26]. We then investigated the kinetics of mt-EFTu^{R336Q} vs. mt-EFTu^{wt} binding activity by using an *in-vitro* non-enzymatic hydrolysis protection assay. This experiment is based on the ability of mt-EFTu to bind aminoacylated mt-tRNAs, in our case in vitro-aminoacylated Ser-mt-tRNA$^{Ser(UCG)}$ and Ser-mt-tRNA$^{Ser(UGA)}$, thus protecting them from spontaneous degradation, which is well demonstrated to occur by the "blank" experiments displayed in Fig. 2. As shown in Fig. 4, mutant mt-EFTu^{R336Q} failed to protect both Ser-mt-tRNA^{Ser}

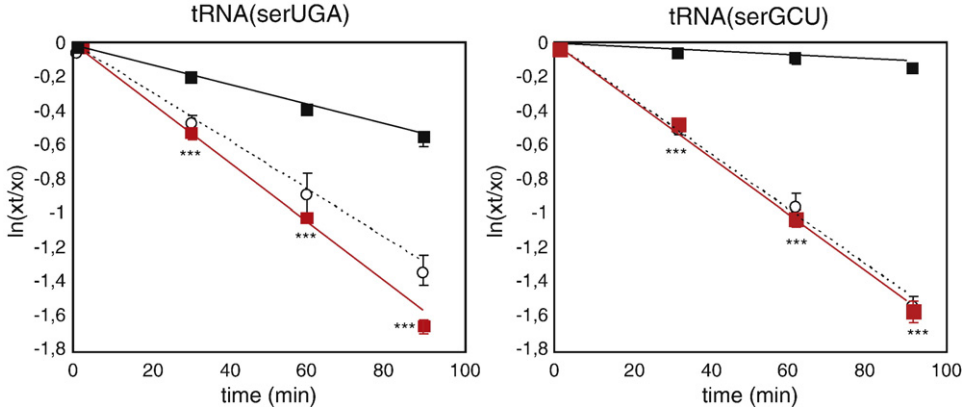

Fig. 4. Non-enzymatic hydrolysis protection assay of Ser-mt-EFTu-tRNA^{Ser}. Time course of $\ln(x_t/x_0)$ values (symbols) and linear regression (lines) of TCA-precipitable [^{3}H]Ser-tRNA$^{Ser(UGA)}$ (left panel) and [^{3}H]Ser-tRNA$^{Ser(GCU)}$ (right panel) as a function of time (see Materials and methods). Closed black squares and continuous line represent values obtained with mt-EFTu^{wt}. Closed red squares and continuous line represent values obtained with mutant mt-EFTu^{R336Q}. Open circles and dotted line represent blank reactions obtained by omitting recombinant mt-EFTu from the reaction mix. Student's t-test analysis of the mt-EFTu^{R336Q} form vs. mt-EFTu^{wt}: ***$p<0.001$.

species, thus indicating impaired binding and failure to form a functionally active ternary complex.

In conclusion, the profound defect of translation associated with the R336Q change in mt-EFTu is not caused by structural abnormalities of the protein, but is rather determined by functional impairment in the interaction with aminoacylated-tRNA, thus preventing translation to proceed. The R336Q mutation is located on a solvent-exposed β-sheet on the outer surface of domain II of mammalian mt-EFTu [11]. This position makes it unlikely that the R336Q mutation can determine a drastic structural rearrangement of the protein, because the interaction with neighbor amino acid residues is minimal. Since domain II constitutes the tRNA-binding site of mt-EFTu [5], we have previously hypothesized that the most probable effect of the R336Q substitution is to hamper the formation of the GTP:EFTu:aminoacyl-tRNA ternary complex. This hypothesis, which was supported by the demonstration that the amount and electrophoretic mobility of mt-EFTu^{R336Q} are both normal [11], is now proven mechanistically by the results of our protection assay.

The virtually complete suppression of mt-EFTu^{R336Q} binding capacity also explains the extreme severity of the recessive syndrome determined by the mutation in humans, although it is surprising that the pathological and clinical features of the disease were restricted to the central nervous system, in spite that the severe mitochondrial translation defect was generalized, and mt-EFTu is an essential constitutive protein expressed in all cells and tissues. Tissue-specificity is an unsolved problem in many mitochondrial disorders, which suggests the presence of complex, still unknown regulatory and control networks. Future work is warranted to clarify this important aspect of mt-EFTu as well as other proteins involved in mitochondrial biogenesis.

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